

METHOD FOR LABELING PHOSPHORYLATED PEPTIDES, METHOD FOR SELECTIVELY ADSORBING PHOSPHORYLATED PEPTIDES, COMPLEX COMPOUNDS USED IN THE METHODS, PROCESS FOR PRODUCING THE COMPLEX COMPOUNDS, AND RAW MATERIAL COMPOUNDS FOR THE COMPLEX COMPOUNDS

5

BACKGROUND OF THE INVENTION

Field of the Invention

10 The present invention relates to a method for labeling phosphorylated peptides, a method for selectively adsorbing phosphorylated peptides, complex compounds usable in the methods, a process for producing the complex compounds, and compounds usable as a raw material in the production process.

15 Description of the Related Art

There are known in vivo enzymes having serine, threonine or tyrosine residue at a specific site corresponding to an active center or allosteric site. The enzymatic activity of these enzymes is controlled by phosphorylating or dephosphorylating hydroxyl group in these residues
20 by an enzyme called kinase and the like. Also, there are known enzymes whose enzymatic activity is controlled by phosphorylating or dephosphorylating an amino group or an imino group in lysin, arginine or histidine, or a carboxyl group in aspartic acids or glutamic acids.

One of the examples of the metabolic systems which are controlled
25 by the aforementioned phosphorylation-dephosphorylation is a system of suppressing synthesis of glycogen and decomposing the same. This metabolic system is primarily cascade-controlled by the phosphorylation-dephosphorylation.

A recent study elucidated that the
30 phosphorylation-dephosphorylation plays a significant role in disease-related metabolic systems.

For instance, it is said that one of the causes of cell carcinogenesis is abnormality in the phosphorylation-dephosphorylation. Specifically, progress and stop of cell cycle are controlled by phosphorylation or
35 dephosphorylation of various enzymes, i.e., proteins. Cycline and

cycline-dependent kinase (CDK) are relevant factors in the phosphorylation or dephosphorylation. If the mechanism relating to cycline and CDK is impaired, phosphorylation or dephosphorylation may be uncontrollable, thereby triggering abnormal proliferation of cells.

5 In addition to the above, facts are known that protein kinase C is related with degranulation of histamine causative of allergic disorders such as atopic dermatitis and pollen allergy, and that phosphorylated tau-protein is causative of neurofibrillary tangle in the brains of Alzheimer's patients.

10 In view of the above, comprehending the condition of phosphorylation-dephosphorylation of proteins provides useful measures not only in investigating expression of genes in living tissue cells and evaluating the enzymatic activity of the cells, but also in diagnosing diseases or medical treatment.

15 The conventional methods for identifying phosphorylated proteins or dephosphorylated proteins have various drawbacks.

For instance, while an enzyme immunoassay is advantageous in analyzing a target protein sample of a very small amount, it is difficult to obtain antibodies of the target protein of a sufficient amount.

20 Further, in case that the level of the target protein is several kDa or lower, it is impossible to prepare an antibody that is securely bonded to a site in the protein where phosphorylation occurs.

There is proposed a method for detecting a protein specifically bonded by a phosphoric acid with use of a phosphoric acid labeled with a radioactive isotope ^{32}P . However, special attention should be paid in handling radioactive isotopes, and appropriate administration and disposal of waste liquid of the radioactive isotopes are required.

25 There is proposed an idea of applying two-dimensional electrophoresis in view of the fact that electric charges are differentiated between phosphorylated proteins and dephosphorylated proteins. However, it is extremely difficult to identify the band or spot of a phosphorylated or dephosphorylated protein in analyzing a sample derived from a living organism, because the sample contains a variety of proteins. Furthermore, use of a radioactive isotope to
35 identify the band or spot involves the aforementioned problems.

The document, Morio YASHIRO, et al. [Preparation and Study of Dinuclear Zinc(II) Complex for the Efficient Hydrolysis of the Phosphodiester Linkage in a Diribonucleotide], Journal of the Chemical Society, Chemical communications, pp.1793-1794 (1995), recites a zinc complex. The zinc complex has a function that two zinc ions in the complex dissociate a phosphoric acid group, namely, phosphoric diester, from dinucleotide. However, the function of the zinc complex disclosed in the document is merely a catalyst. The document does not disclose the ability of the zinc complex to bond coordinately to a phosphoric acid group. The experiments conducted by the inventors reveal that a dissociation constant of the zinc complex to a phosphoric acid group sandwiched by two nucleosides, namely, a phosphoric diester, is extremely high. In other words, the zinc complex has a low coordinatability to a phosphoric diester moiety.

Further, the document, Hidekazu ARII, et al., [A novel diiron complex as a functional model for hemerythrin], Journal of Inorganic Biochemistry, 82, pp.153-162 (2000), recites an iron complex having a structure analogous to the structure of the zinc complex. The iron complex, however, is a product synthesized as a model of hemerythrin, namely, a carrier protein carrying oxygen molecules. As is the case with the above mentioned document, this document neither discloses nor remotely suggests coordinate bond of the iron complex to a phosphoric monoester moiety.

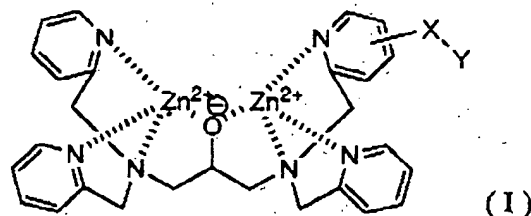
BRIEF SUMMARY OF THE INVENTION

In view of the above, it is an object of the present invention to overcome the problems residing in the prior art. It is another object of the present invention to provide a method for labeling phosphorylated peptides, namely, proteins, for easy detection, and a method for selectively adsorbing the phosphorylated peptides for purification or the like.

It is still another object of the present invention to provide compounds that are capable of being highly coordinated to the phosphorylated peptides and usable in the labeling method and the selective adsorbing method, a process for producing the compounds, and

raw material compounds usable in the production process.

According to an aspect of the present invention, provided is a method for labeling a phosphorylated peptide by a complex compound represented by the formula (I):



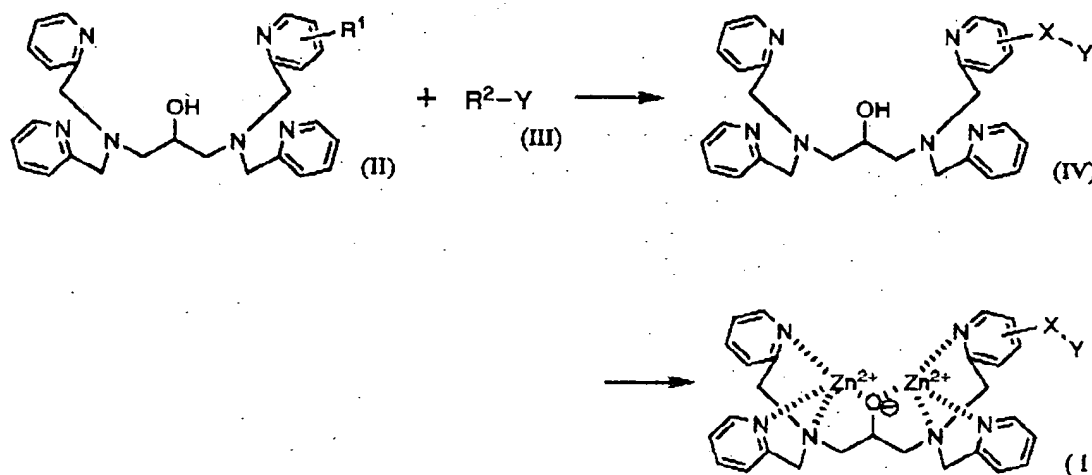
5 wherein X is a linker moiety, and Y is a labeling group.

According to another aspect of the present invention, provided is a method for selectively adsorbing a phosphorylated peptide by using the above complex compound.

10 According to yet another aspect of the present invention, provided is the above complex compound.

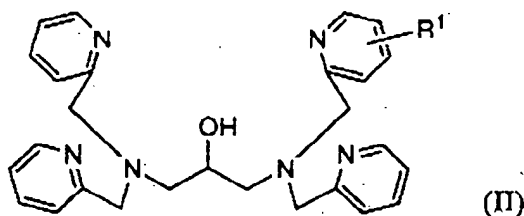
According to still another aspect of the present invention, provided is a process for producing the compound (I), comprising Scheme 1.

Scheme 1.



15 wherein R^1 and R^2 each is a reactive group for forming the linker moiety X, and Y is a labeling group.

According to still another aspect of the present invention, provided is a compound represented by the formula (II):



wherein R^1 is a reactive group except an aminomethyl group, a hydroxymethyl group, an amino group, and a carboxyl group.

According to the inventive labeling method, the phosphorylated peptide, namely, a protein can be easily detected. Thus, the present invention is useful in diagnosing diseases or the like with use of samples derived from living organisms or the like.

Further, since the inventive compound (I) shows a unique coordination bond to the two hydroxy groups in a phosphoric monoester moiety or phosphoric ion, the inventive compound (I) is useful as a compound usable in the inventive method. Additionally, the inventive compound (I) is useful for purifying or concentrating phosphorylated peptide and obtaining the chemical information of phosphorylated peptide.

These and other objects, features and advantages of the present invention will become more apparent upon reading of the following detailed description along with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is an illustration showing a result of examination by an MALDI-TOF mass spectrometer regarding a zinc complex according to the present invention.

FIG. 2 is an illustration showing electrophoresis gels, which is dyed by the inventive zinc complex (A) and further conventional dye (B). This illustration makes it clear that the inventive method can identify only phosphorylated peptide, while conventional method dyes all of the peptide.

FIG. 3 is an illustration showing a result of examination by the MALDI-TOF mass spectrometer regarding a zinc complex according to the present invention, as well as an enlarged illustration showing a certain region of the examination result.

FIG. 4 is an illustration showing a result of examination by the MALDI-TOF mass spectrometer regarding a zinc complex according to the present invention, as well as an enlarged illustration showing a certain region of the examination result.

5 FIG. 5 is an illustration showing a result of examination by the MALDI-TOF mass spectrometer regarding the raw material compound used in producing the inventive compound having biotin as a labeling group, as well as an enlarged illustration showing a certain region of the examination result.

10 FIG. 6 is an illustration showing a result of examination by the MALDI-TOF mass spectrometer regarding the raw material compound used in producing the inventive compound having biotin as a labeling group, as well as an enlarged illustration showing a certain region of the examination result.

15 FIG. 7 is an illustration showing a result of examination by the MALDI-TOF mass spectrometer regarding a zinc complex according to the present invention, as well as an enlarged illustration showing a certain region of the examination result.

20 FIG. 8 is an illustration showing a result of examination by the MALDI-TOF mass spectrometer regarding a zinc complex according to the present invention.

25 FIG. 9 is an illustration showing electrophoresis gels, which is dyed by the inventive zinc complex (A) and further conventional dye (B). This illustration makes it clear that the inventive method can identify only phosphorylated peptide, while conventional method dyes all of the peptide.

DETAILED DESCRIPTION OF THE INVENTION

30 A primary feature of the inventive method resides in that a phosphorylated peptide can be easily identified by forming a composite compound in which a complex compound having a labeling group as represented by the formula (I) is specifically bonded to the phosphorylated peptide.

35 There have been known various metallic complexes capable of being bonded to a phosphoric acid group. However, a compound which is analogous to the compound represented by the formula (I) and has a labeling group

has been unknown. The inventors found that use of the complex compound represented by the formula (I) is advantageous in easily detecting and identifying a phosphorylated peptide even in a sample of a living organism containing a multitude of kinds of peptides, and accomplished the present invention.

In the following, a method for labeling phosphorylated peptides according to an embodiment of the present invention is described.

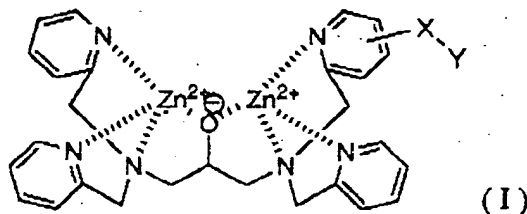
First, prepared is a sample containing substantially all the possible kinds of peptides constituting a tissue cell to be examined. The preparation can be conducted according to a conventional method practiced in the biochemistry.

Next, the peptides contained in the sample are separated. The separation method is not specifically limited, and a conventional separation method such as electrophoresis can be applied.

In case of implementing the electrophoresis, the gel after the electrophoresis is immersed in a solution containing the complex compound represented by the formula (I) to label the phosphorylated peptide, and then, the phosphorylated peptide is detected by a detecting method depending on the kind of the labeling group.

A solvent usable in the solution containing the complex compound represented by the formula (I) is not specifically limited, as far as the solvent does not hinder detection of the phosphorylated peptide. Water including a buffer and a solution containing a salt other than a buffer; alcohols such as methanol and ethanol; and a mixed solvent containing these components are examples of the solvent. Preferably, an aqueous solvent is used primarily for the purpose of preventing denaturation of the peptide.

Next, the compound (I) used in the above method is described.



wherein X is a linker moiety, and Y is a labeling group.

In the formula (I), Zn is selected as a coordinate metal because Zn is highly coordinated to a phosphoric acid group in a phosphorylated protein.

namely, a phosphoric monoester moiety.

The linker moiety in the present specification and the claims means a group capable of linking a main skeleton and a labeling group. The linker moiety facilitates production of the compound (I), and inhibits the labeling group from hindering coordination of the compound (I) to the phosphoric acid group bonded to the peptide. In view of this, the linker moiety may be a coordination bond which directly links the main skeleton and the labeling group if it is easy to obtain a raw material compound in which a labeling group is directly linked to a main skeleton in synthesizing the compound (I), or if the labeling group is of such a small size that hindrance of coordination of the compound (I) to the phosphoric acid group is securely prevented.

The kind of the linker moiety in the present specification and the claims is not specifically limited, as far as the linker moiety has the above functions. Examples of the linker moiety are: a C1-C6 alkylene group, an amino group (-NH-), an ether group (-O-), a thioether group (-S-), a carbonyl group (-C(=O)-), a thionyl group (-C(=S)-), an ester group, an amide group, a urea group (-NHC(-O)NH-), a thiourea group (-NHC(=S)NH-); a C1-C6 alkylene group having, at one end thereof, a group selected from the group consisting of an amino group, an ether group, a thioether group, a carbonyl group, a thionyl group, an ester group, an amide group, a urea group, and a thiourea group; a C1-C6 alkylene group having, at the opposite ends thereof, two groups selected from the group consisting of an amino group, an ether group, a thioether group, a carbonyl group, a thionyl group, an ester group, an amide group, a urea group, and a thiourea group, wherein the groups at the opposite ends are identical to or different from each other; and a group in which two or more than two groups selected from the group consisting of an amino group, an ether group, a thioether group, a carbonyl group, a thionyl group, an ester group, an amide group, a urea group, a thiourea group, and a C1-C6 alkylene group are linearly linked.

The C1-C6 alkylene group means a bivalent aliphathic hydrocarbon group having 1 to 6 carbon atoms of a straight chain or a branched chain, such as methylene, ethylene, propylene, tetramethylene, hexamethylene, methylethylene, methylpropylene, and dimethylpropylene, and preferably is a C1-C4 alkylene group, and more preferably is a C1-C2 alkylene group.

The labeling group in the present specification and the claims is not specifically limited, as far as it is generally used in the biochemistry. However, a compound containing a radioisotope is not preferable in the aspect of handling. Examples of the labeling group are a fluorescent group,
5 a group containing a nitro oxide radical and biotin.

The fluorescent group is a substituent capable of stably generating fluorescence of a relatively long wavelength, and a group generally used in the biochemistry can be unlimitedly used as the fluorescent group, irrespective of the property that the compound is soluble in water or oil.

10 Examples of the fluorescent group are aminomethylcoumarin and its derivatives, fluoroscein and its derivatives, tetramethylrhodamine and its derivatives, anthraniloyl and its derivatives, nitrobenzoxadiazole and its derivatives, and dimethylaminonaphthalene and its derivatives.

The group containing a nitro oxide radical is a group having a stable
15 radical, and is capable of detecting a phosphorylated peptide by electron spin resonance (ESR). Generally, a biological molecule does not show electron spin resonance because it does not have an unpaired electron. On the other hand, since a peptide bonded by the compound (I) having a nitro oxide radical being coordinated thereto shows electron spin resonance,
20 the phosphorylated peptide is identifiable.

Biotin has specific and high affinity to avidin derived from albumen and streptoavidin derived from actinomycetes. In view of this, the inventive complex compound can be specifically bonded to an enzyme by way of biotin and avidin or streptoavidin, by bonding avidin or streptoavidin
25 to the inventive compound (I) having biotin as a labeling group and further bonding a biotinated enzyme. The phosphorylated peptide can be identified by using the enzyme such as alkaliphosphotase, peroxidase and luciferase, and by using a coloring reagent depending on the kind of the enzyme. For instance, if first phosphorylated peptide is bonded by the inventive
30 complex compound, then the complex compound is bonded by alkaliphosphotase as the enzyme via streptoavidin, further nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as the coloring reagents are added, and the mixture is reacted for several hours, the phosphorylated peptide turns into purple, thereby making it possible to identify the
35 phosphorylated peptide. Further, streptoavidin labeled with a

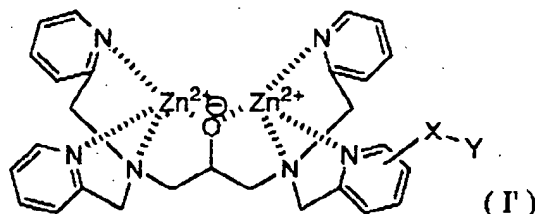
fluorescent pigment such as rhodamine is commercially available. Use of the streptoavidin labeled with the fluorescent pigment makes it possible to identify a phosphorylated peptide by a known fluorescent image analyzing method.

5 Next, a method for selectively adsorbing phosphorylated peptides according to an embodiment of the present invention is described.

 According to the method for selectively adsorbing phosphorylated peptides, biotin or the like capable of specifically being bonded to a specific compound is used as a labeling group. For instance, an agarose
10 gel with streptoavidin being bonded thereto is commercially available. An agarose gel with the inventive complex compound being bonded thereto can be obtained by applying the inventive complex compound having biotin as a labeling group to an agarose gel for reaction. The phosphorylated peptide in a mixed sample can be selectively adsorbed to the inventive
15 complex compound by applying the mixed sample to the agarose gel. After the selective adsorption, adding a phosphoric acid buffer or the like capable of desorbing the phosphorylated peptide from the inventive complex compound makes it possible to obtain the phosphorylated peptide exclusively. In this way, using the agarose gel is advantageous in purifying or
20 concentrating a phosphorylated peptide without electrophoresis. Also, magnetic beads with streptoavidin being bonded thereto are commercially available. Use of the magnetic beads also makes it possible to purify or concentrate a phosphorylated peptide. Further, plates with streptoavidin being bonded thereto are commercially available for measuring surface
25 plasmon resonance (SPR). A plate with the inventive complex compound being bonded thereto for measuring surface plasmon resonance can be obtained by applying the inventive complex compound having biotin as a labeling group onto the plate. Presence or absence of the phosphorylated peptide in a sample can be detected by measuring the surface plasmon resonance
30 (SPR) of the plate.

 It is possible to synthesize a compound equivalent to the compound (I), in which a methyl group or the like is introduced to a pyridine ring, to provide substantially the same operations and effects as in the embodiment of the present invention. Such a compound equivalent to the
35 compound (I) is embraced in the scope of the present invention.

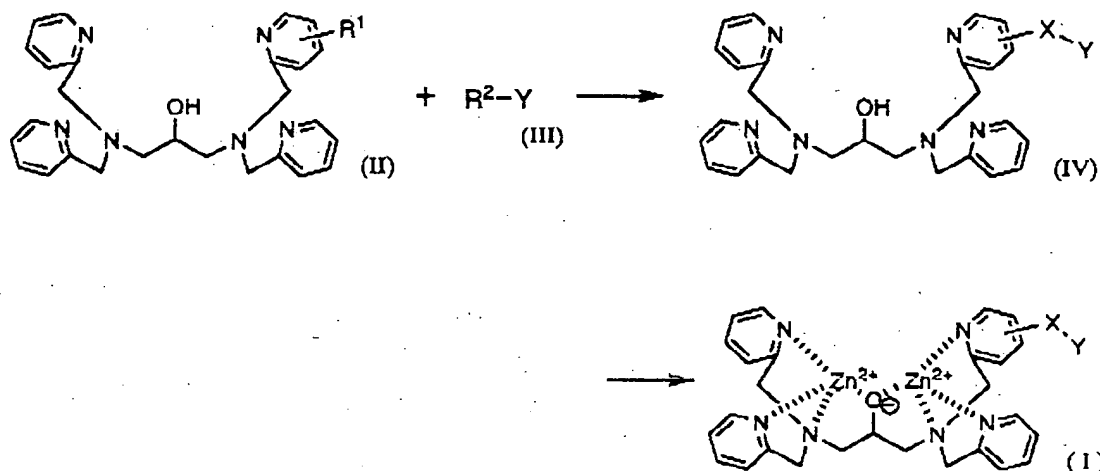
The location of the (-X-Y) group in the inventive compound is not specifically limited. The (-X-Y) group may be located at the site as shown in the compound (I').



5 The compound (I) and the compound (I') are substantially equivalent to each other. Although it is not clear as to which compound is synthesized, the compound (I) or the compound (I'), what is actually synthesized is conceivably a synthesized mixture of the compound (I) and the compound (I'). It is needless to say that the compound (I') is embraced in the scope
10 of the present invention.

A complex compound represented by the formula (I) can be easily produced by a process comprising Scheme 1.

Scheme 1



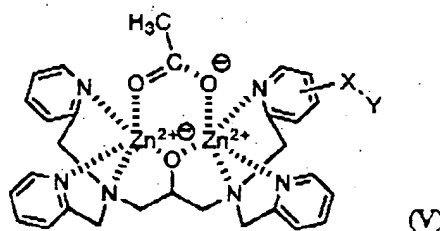
wherein X and Y is the same as defined above, and R¹ and R² each is a reactive
15 group for forming the linker moiety X.

In the above Scheme, the labeling group Y is introduced to the main skeleton via the linker moiety X by reacting the reactive groups R¹ and R².

The kind of R¹, R², the solvent, a reaction temperature, a reagent
20 other than the above, a purification method, and other factors are primarily

determined by the kind of X. For instance, in case of introducing a labeling group via an amino group (a secondary or tertiary amino group), as a combination of R¹ and R², a combination of a group having an amino group (a primary or a secondary amino group) at a distal end thereof and a
 5 elimination group such as a halogen atom. Condensing R¹ and R² under the presence of basic groups in the solvent is an example of general reaction condition. In case that R¹ is an active group, it is very easy to introduce a labeling group.

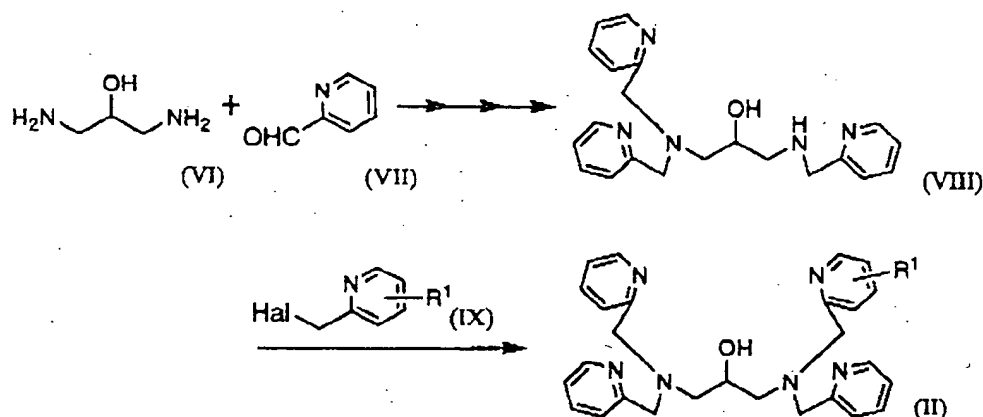
Next, the compound (I) can be synthesized by adding a metallic salt
 10 to a solution containing the compound (IV). Zinc (II) nitrate or zinc (II) acetate may be added as the metallic salt. In case that zinc (II) acetate is added, a compound represented by the following formula (V) in which acetic acid is temporarily coordinated is produced.



The compound (V) is chemically more stable than the compound (I), and
 15 accordingly useful in storage. The compound (V) is equivalent to the compound (I), and is usable in the similar manner as the compound (I). Specifically, by adding the compound (V) to the mixture containing the peptide, the phosphorylated peptide can be detected because the phosphoric monoester moiety is interchangeably coordinated to the compound (I) in
 20 place of the acetic acid.

The compound (II), namely, a raw material compound of the compound (I) can be synthesized by the following Scheme 2.

Scheme 2



wherein R^1 is the same as defined above, and "Hal" is a halogen atom, and preferably is a bromine.

The compound (VI), i.e., 1,3-diamino-2-propanol, as a raw material compound may be commercially available. Further, since both the compound (VII) and the compound (IX) have a relatively simple structure, the compound (VII) and (IX) may be commercially available, or can be synthesized by a well-known method for a person skilled in the art.

In Scheme 2, first, the compound (VI) and the compound (VII) are reacted with each other under the presence of a catalyst for condensation to yield the compound (VIII). This reaction may be implemented step by step by introducing the compound (VII). Alternatively, the compound (VIII) can be obtained by a single step by using 3 or more equivalents of the compound (VII) to the compound (VI).

In Scheme 2, reductive amination is carried out as a condensation reaction. A solvent used in the reductive amination is not specifically limited, as far as the solvent is capable of substantially dissolving the compound (VI) and the compound (VII), and does not inhibit the amination. For instance, alcohols such as methanol, ethanol and isopropanol; ethers such as diethyl ether, tetrahydrofuran and dioxane; water; or a mixed solvent containing two or more of these components can be used as the solvent.

The reductive amination can be carried out with use of a conventional reducing reagent after condensing the compound (VI) and the compound (VII) under the presence of concentrated hydrochloric acid, as a catalyst.

An optimal condition regarding the reaction temperature and the reaction time can be optionally selected depending on the kind of the raw

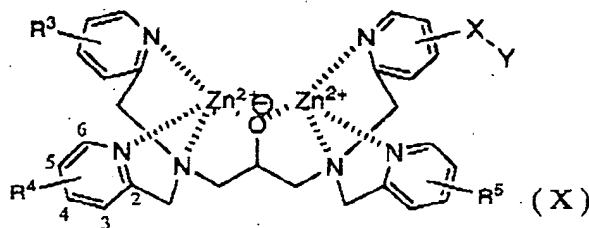
material compound or other factors. For example, the reaction may be carried out at a reaction temperature from 20 to 80°C for a reaction time from 12 to 100 hours.

After the reaction is completed, the solvent and the like are distilled off under depressurization before adding water. After water is added, the resultant mixture is extracted with a water-insoluble solvent, and the organic layer is dried over anhydrous magnesium sulfate or the like. Thereafter, the solvent is distilled off under depressurization. Subsequently, the residue is purified by a well known process such as silica gel column chromatography, thereby to yield the compound (VIII).

The process for yielding the compound (VIII) is not limited to the process as shown by Scheme 2. Alternatively, the compound (VIII) may be synthesized using the compound (VI) and a halogen compound, for example.

Next, the compound (II) can be synthesized by reacting the compound (VIII) with the compound (IX). This reaction can be carried out by a known process of synthesizing tertiary amines. For instance, the compound (VIII) and the compound (IX) are condensed under the presence of base in a solvent. In the condensing step, a protective group may be introduced and cleaved depending on the kind of R¹ according to needs. Alternatively, the compound (II) may be synthesized by implementing the condensing step with use of a compound having an inactive substituent group in place of using R¹ in the compound (IX), and by substituting R¹ for the inactive substituent group by functional group conversion. For instance, the condensing step is implemented by using a compound having a nitro group as the inactive substituent group and by substituting an amino group as the reactive group for the nitro group.

The following complex compound (X) can be used as a compound usable in the inventive method, in place of the complex compound (I).



wherein X, Y is the same as defined above, and R³ through R⁵ each is an electron donating substituent group at the 4 or 6 position on the pyridine

ring.

The complex compound (X) used in the inventive method is electrically enriched with pyridine nitrogen by the electron donating substituent group that has been introduced to an appropriate position for substitution.

5 Accordingly, the complex compound (X) used in the inventive method is highly coordinated to zinc, thereby making it possible to produce the complex compound (X) easily, while providing stability.

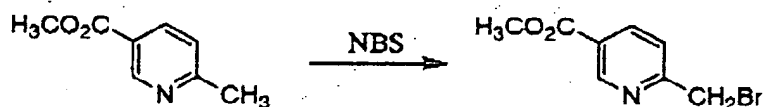
10 A manner of using the complex compound (X), a process for producing the complex compound (X), and a raw material compound for the complex compound (X) are substantially the same as those regarding the complex compound (I).

15 In the above methods and compound (I), it is preferable to use biotin, as the labeling group, in producing the complex compound. Use of biotin is preferred because biotin is easy to be handled, and has high usability in the point that it exhibits various coloring reactions. Use of biotin is effective in easily identifying the phosphorylated peptide.

20 In the following, production examples and experiment examples are illustrated to describe the present invention in detail. The present invention is, however, not limited to the illustrated examples.

EXAMPLES

Production Example 1-1: Methyl 6-bromomethylnicotinate



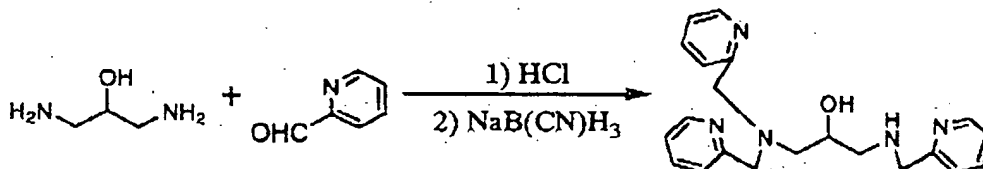
25 To a solution of methyl 6-methylnicotinate (50g, 331mmol) in carbon tetrachloride (625mL), was added N-bromosuccinimide (59g, 331mmol). Further 1.0g of benzoyl peroxide was added, the mixture was reacted at a temperature from 40 to 50°C for 24 hours with irradiation of light from a projector.

30 After the reaction mixture was cooled, the precipitated crystals were separated by filtration. The filtrate was washed with an aqueous solution containing sodium hydrogencarbonate, and concentrated. The residue obtained by the concentration was purified by silica gel column

chromatography, thereby to yield 37g of the target compound.

¹H-NMR (CDCl₃, 300MHz) : δ 3.96(3H, s, OCH₃), 4.58(2H, s, CH₂Br), 7.54(1H, d, Py), 8.30(1H, dd, Py), 9.17(1H, d, Py)

5 Production Example 1-2: N,N,N'-Tri(2-pyridylmethyl)-1,3-diaminopropane-2-ol

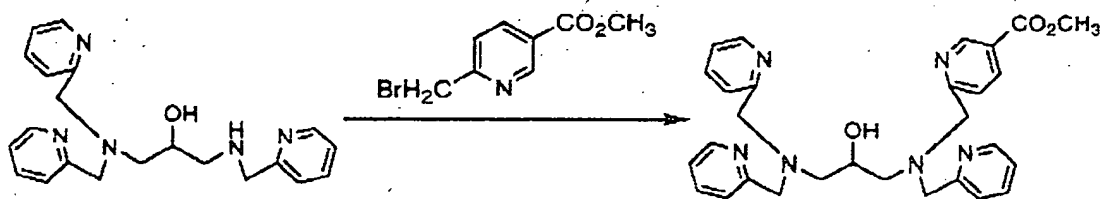


To a solution of 1,3-diaminopropane-2-ol (32.6g, 362mmol) in methanol (2400mL), was added 60mL of concentrated hydrochloric acid. Further 2-pyridine aldehyde (116.3g, 1.09mol) was added dropwise, and then sodium cyanoborohydride (50.16g, 798mmol) was added. After the addition was completed, the mixture was reacted at room temperature for 3 days.

After concentrated hydrochloric acid was added to the solution and the pH of the solution was adjusted to 6, the resulting solution was concentrated to some extent. Then, 0.1N aqueous solution of sodium hydroxide was added to adjust the pH of the solution to 7, followed by extraction with chloroform. The extracts were collected and dried, the resultant was concentrated. The residue obtained by the concentration was purified by silica gel column chromatography, thereby to yield 34g of the target compound.

20 ¹H-NMR (CDCl₃, 300MHz) : δ 2.59-2.83(4H, m, CH₂), 3.86-4.01(7H, m, NCH₂Py, CH), 7.15(3H, dd, Py), 7.23-7.32(3H, m, Py), 7.56-7.65(3H, m, Py), 8.53(3H, dd, Py)

25 Production Example 1-3: N,N,N'-Tri(2-pyridylmethyl)-N'-(5-methoxycarbonyl-2-pyridylmethyl)-1,3-diaminopropane-2-ol



N,N,N'-tri(2-pyridylmethyl)-1,3-diaminopropane-2-ol (18.2g, 50mmol)

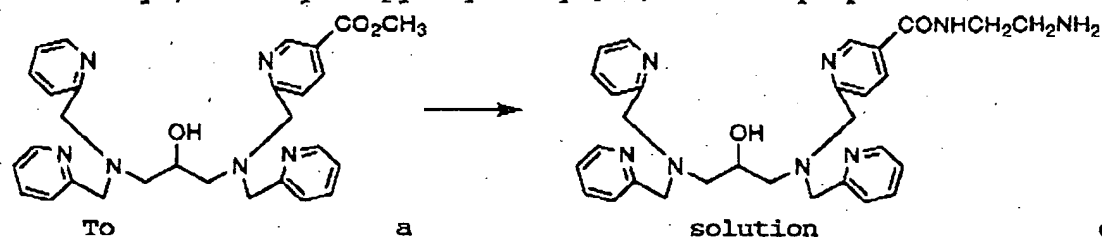
obtained in Production Example 1-2 in dried dimethylformamide (150mL), was added potassium carbonate (13.8g, 100mmol), followed by addition of a solution of the methyl 6-bromomethylnicotinate (11.5g, 50mmol) obtained in Production Example 1-1 in dried dimethylformamide (75mL) dropwise.

5 After the dropwise addition was completed, the mixture was reacted at 50°C for 1 hour.

After the reaction was completed, the solution was cooled. Then, the cooled solution was poured into 750mL of water, and the pH of the solution was adjusted to 8 by adding 1N hydrochloric acid. After extraction with ethyl acetate, the extracts were collected, washed with water and brine, and concentrated. The residue obtained by the concentration was purified by silica gel column chromatography, thereby to yield 21.5g of the target compound.

¹H-NMR (CDCl₃, 300MHz) : δ 2.58-2.73(4H, m, CH₂), 3.83-3.95(12H, m, OCH₃, NCH₂Py, CH), 7.10-7.14(3H, m, Py), 7.34(3H, d, Py), 7.50-4.60(4H, m, Py), 8.17(1H, dd, Py), 8.50(3H, d, Py), 9.09(1H, d, Py)

Production Example 1-4: N,N,N'-Tri(2-pyridylmethyl)-N'-[5-N''-(2-aminoethyl)carbamoyl-2-pyridylmethyl]-1,3-diaminopropane-2-ol



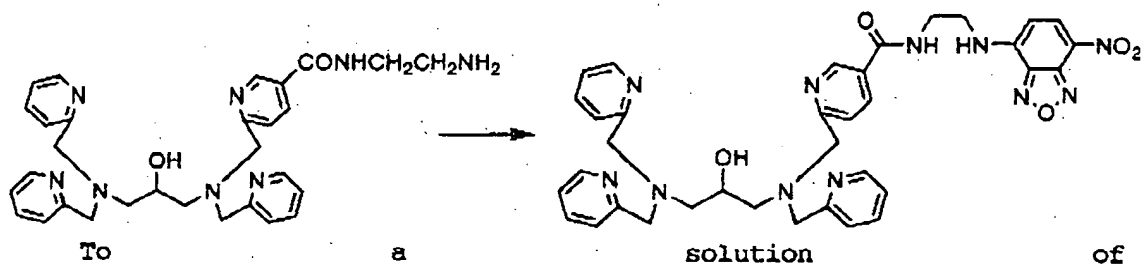
N,N,N'-tri(2-pyridylmethyl)-N'-[5-methoxycarbonyl-2-pyridylmethyl]-1,3-diaminopropane-2-ol (9.7g, 18.9mmol) obtained in Production Example 1-3 in methanol (100mL), was added ethylenediamine (22.7g, 378mmol) dropwise. After the dropwise addition, the mixture was reacted at room temperature for 3 days.

After the reaction was completed, the solution was concentrated, and the residue obtained by the concentration was purified by silica gel column chromatography, thereby to yield 9.72g of the target compound.

¹H-NMR (CDCl₃, 300MHz) : δ 2.54-2.71(4H, m, CH₂), 2.94(2H, t, CH₂N), 3.49(2H, dt, CH₂N), 3.80-3.99(9H, m, NCH₂Py, CH), 7.12(3H, ddd, Py), 7.35(3H, d, Py), 7.45(1H, d, Py), 7.58(3H, ddd, Py), 8.02(1H, dd,

Py), 8.49(3H, ddd, Py), 8.89(1H, d, Py)

Production Example 1-5: N,N,N'-Tri(2-pyridylmethyl)-N'--[5-N''-(7-nitro-2,1,3-benzoxadiazole-4-ylaminoethyl)carbamoyl-2-pyridylmethyl]-1,3-diaminopropane-2-ol

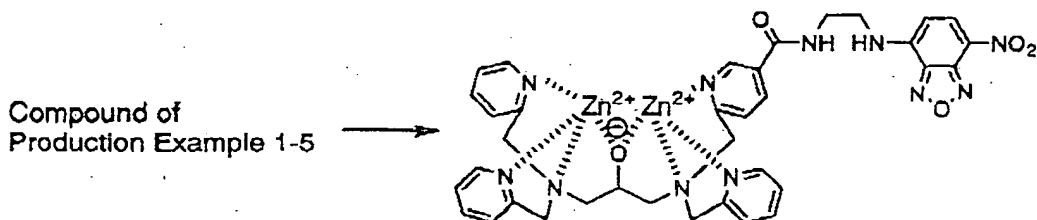


N,N,N'-tri(2-pyridylmethyl)-N'--[5-N''-(2-aminoethyl)carbamoyl-2-pyridylmethyl]-1,3-diaminopropane-2-ol (200mg, 0.37mmol) obtained in Production Example 1-4 in acetonitrile (20mL), was added sodium hydrogencarbonate (336mg, 4.0mmol), followed by addition of 4-chloro-7-nitro-2,1,3-benzoxadiazole (73.8mg, 0.37mmol). The mixture was reacted at room temperature for 2 hours.

After the reaction was completed, the solution was concentrated. Then, 50mL of dichloromethane and 50mL of water were added, and the organic layer and aqueous layer were separated. The organic layer was dried over anhydrous sodium sulfate, concentrated, and the obtained residue was purified by silica gel column chromatography, thereby to yield 71.2mg of the target compound.

¹H-NMR (CDCl₃, 300MHz) : δ 2.46-2.69(4H, m, CH₂), 3.65-3.95(13H, m, NCH₂CH₂N, NCH₂Py, CH), 6.06(1H, d, Ar), 7.08-7.13(3H, m, Py), 7.32(3H, d, Py), 7.42(1H, d, Py), 7.56(3H, ddd, Py), 7.96(1H, dd, Py), 8.44-8.48(3H, m, Py), 8.19(1H, d, Ar), 8.83(1H, d, Py)

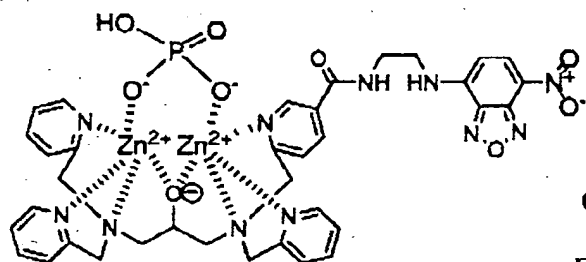
Production Example 1-6: Solution containing the inventive zinc complex



Prepared was 50μM aqueous solution containing the compound obtained

in Production Example 1-5, followed by addition of zinc nitrate of 2 equivalents to the solution. Thus, a solution containing the inventive zinc complex was prepared.

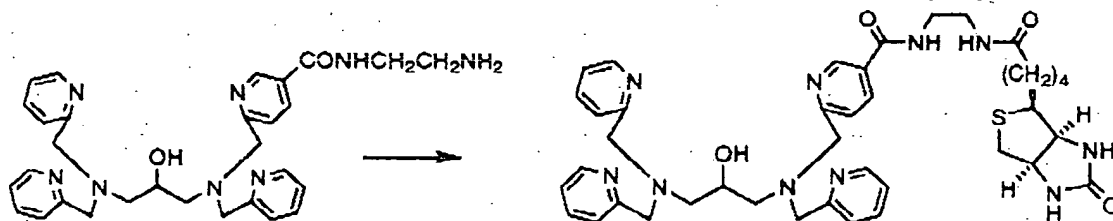
The zinc complex was identified according to the following method. Specifically, the compound obtained in Production Example 1-5 was dissolved in a phosphoric acid buffer (pH=6.86) to obtain the 50 μ M solution, followed by addition of zinc nitrate of 2 equivalents to the solution. The inventive zinc complex in the solution exhibits the following structure, and was identified by MALDI-TOF mass spectrometer (Matrix: 2',4',6'-trihydroxyacetophenone, Mode: reflector, Accelerating Voltage: 20000V, Grid Voltage: 57.500%, Laser: 2500, Scans Averaged: 128, Pressure: 5.05e-07).



$C_{36}H_{37}N_{11}O_9PZn_2$
Mol. Wt.: 929.49
Exact Mass: 926.11

The result of measurement by the MALDI-TOF mass spectrometer is shown in FIG.1. As shown in FIG.1, a molecular ion peak at 926.2 (exact mass: 926.11) was observed. It is considered that a molecular ion peak at 910.2 appeared because the oxygen in the oxadiazole group was eliminated.

Production Example 2-1: N,N,N'-Tri(2-pyridylmethyl)-N'--[5-N''-(2-D-biotinamidoethyl)carbamoyl-2-pyridylmethyl]-1,3-diaminopropane-2-ol



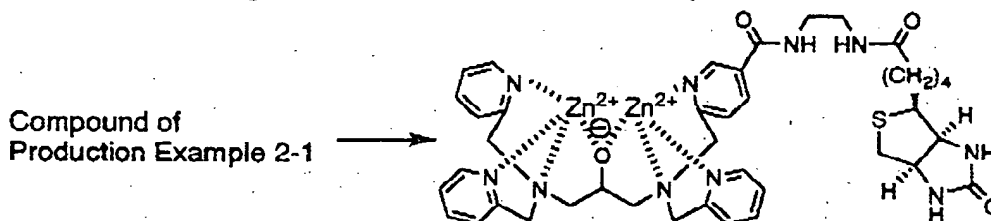
To a solution of D-biotin (137mg, 0.56mmol) in dimethylformamide (10mL), was added 1,1'-carbonyldiimidazole (116mg, 0.72mmol). The mixture was reacted at room temperature for 12 hours. Thereafter, the solution was cooled with ice, followed by dropwise addition of a solution of N,N,N'-tri(2-pyridylmethyl)-N'--[5-N''-(2-aminoethyl)carbamoyl-2-

pyridylmethyl]-1,3-diaminopropane-2-ol (260mg, 0.48mmol) obtained in Production Example 1-4 in dimethylformamide (3mL). The cooling bath was detached, and the reaction was carried out at room temperature for 2 hours.

After the reaction was completed, the solution was poured into 50mL of water, followed by extraction with 50mL of chloroform twice. After the extracts were concentrated, the crude product was purified by silica gel column chromatography, thereby to yield 265mg of the target compound.

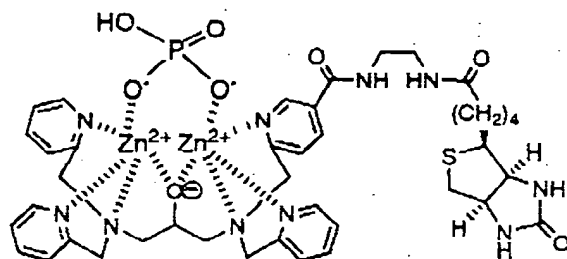
¹H-NMR (CDCl₃, 300MHz) : δ 1.27-1.47(2H, m, CH₂), 1.50-1.75(4H, m, CH₂), 2.13-2.26(2H, m, COCH₂), 2.52-2.74(5H, m, NCH₂, SCH₂), 2.79-2.88(1H, m, SCH₂), 3.01-3.12(1H, m, SCH), 3.43-3.65(4H, m, NCH₂CH₂N), 3.80-4.02(9H, m, NCH₂Py, CHO), 4.22-4.28(1H, m, NCH), 4.42-4.49(1H, m, NCH), 5.83(1H, bs, NHCO), 6.60(1H, bs, NHCO), 7.07-7.18(3H, m, Py), 7.31-7.38(3H, m, Py), 7.44(1H, d, Py), 7.59(3H, ddd, Py), 8.03(1H, dd, Py), 8.15(1H, bs, NHCO), 8.42-8.58(3H, m, Py), 8.94(1H, d, Py)

Production Example 2-2: Solution containing the inventive zinc complex



The compound obtained in Production Example 2-1 was dissolved in a phosphoric acid buffer (pH=6.86) to obtain 3mM solution, followed by addition of zinc nitrate of 2 equivalents to the solution.

The inventive zinc complex in the solution exhibits the following chemical structure, and was identified by the MALDI-TOF mass spectrometer (Matrix: 2',4',6'-trihydroxyacetophenone).



C₄₀H₅₀N₁₀O₈PSZn₂
Mol. Wt.: 992.69
Exact Mass: 989.19

A result of measurement by the MALDI-TOF mass spectrometer is shown

in FIG.3. As shown in FIG.3, a molecular ion peak at 989.6 (exact mass: 989.19) is observed.

Experimental Example 1: 10% Native-polyacrylic amide gel electrophoresis

5 First, prepared were gels for electrophoresis, a pH buffer for electrophoresis, and a coloring solution for dissolving samples under the following conditions.

Stacking gel:

125mM of Tris-hydrochloric acid buffer (pH=6.8)

10 4.5% (w/v) of polyacrylamide (acrylamide : bisacrylamide = 30:1)

Separation gel:

375mM of Tris-hydrochloric acid buffer (pH=8.8)

10% (w/v) of polyacrylamide (acrylamide : bisacrylamide = 30:1)

15 pH buffer for electrophoresis (pH=8.3):

25mM of Tris

190mM of glycine

Coloring solution for dissolving samples (3-times concentrated solution):

20 195mM of Tris-hydrochloric acid buffer (pH=6.8)

10% (w/v) of glycerol

0.1% (w/v) of bromo phenol blue (BPB), as a coloring marker used in electrophoresis

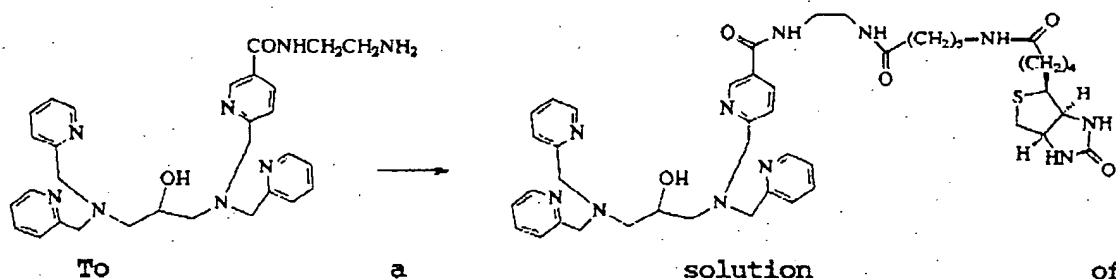
25 Next, 2 μ g each of 1: Bovine Serum Albumin, 2: β -casein (phosphorylated), and 3: β -casein (de-phosphorylated) was dissolved in the coloring solution to prepare samples. The respective samples were plotted on the gel. Then, a constant electric current of 4mA was applied until the coloring marker was flowed out.

30 The gel was immersed in the zinc-complex-containing solution (50 μ M) obtained in Production Example 1-6 for about 30 minutes. Then, the gel was taken out from the solution, and photographed under irradiation by a UV lamp. Further, the gel was dyed with Coomassie brilliant blue according to a conventional dying process, and the dyed gel was photographed. The gel dyed with the zinc-complex-containing
35 solution is referred to as "gel A", and the gel dyed with Coomassie

brilliant blue is referred to as "gel B", both of which are shown in FIG.2.

As is obvious from FIG.2, according to the inventive method, β -casein being bonded with phosphoric acid can be identified exclusively. Thus, it is clear that the inventive method is advantageous in identifying phosphorylated peptides exclusively in samples derived from living organisms.

Production Example 3-1: N,N,N'-Tri(2-pyridylmethyl)-N'-[5-N'-2-(6-D-biotinamidohexacarboxyamidoethyl)carbamoyl-2-pyridylmethyl]-1,3-diaminopropane-2-ol



To a solution of N,N,N'-tri(2-pyridylmethyl)-N'-[5-N'-2-(6-D-biotinamidohexacarboxyamidoethyl)carbamoyl-2-pyridylmethyl]-1,3-diaminopropane-2-ol (113mg, 0.21mmol) obtained in Production Example 1-4 in acetonitrile (10mL), was added a solution of 5-(N-succineimidyloxycarbonyl)pentyl-D-biotinamide (95mg, 0.21mmol) in dimethylsulfoxide (2mL) dropwise.

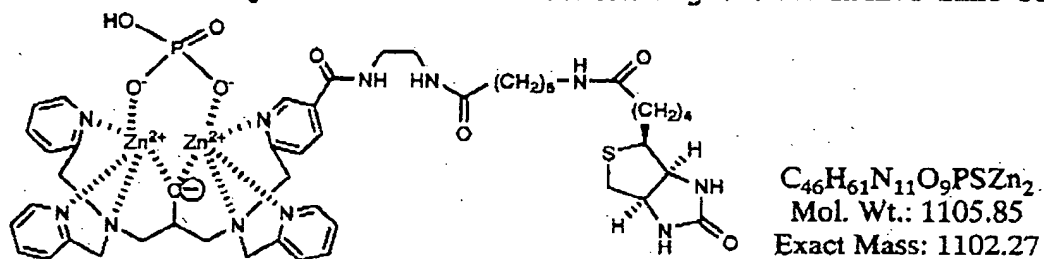
After the mixture was reacted at room temperature for 6 hours, the reaction mixture was concentrated, and the crude product obtained by the concentration was purified by silica gel column chromatography, thereby to yield 136mg of a target compound (yield: 76%).

$^1\text{H-NMR}$ (CDCl_3 , 500MHz): δ 1.25-1.32(2H, m, CH_2), 1.38-1.47(4H, m, CH_2), 1.56-1.67(5H, m, CH_2), 1.68-1.77(1H, m, CH_2), 2.11-2.22(4H, m, COCH_2), 2.58(2H, dd, $J=8.0$ and 13.3Hz , NCH), 2.66(1H, dd, $J=3.9$ and 13.3Hz , NCH), 2.68(1H, dd, $J=3.9$ and 13.3Hz , NCH), 2.71(1H, dd, $J=3.0$ and 13.3Hz , SCH), 2.88(1H, dd, $J=5.2$ and 13.0Hz , SCH), 3.07-3.21(3H, m, NCH, SCH), 3.45-3.59(4H, m, NCH_2), 3.82-3.91(8H, m, NCH_2Py), 3.94(1H, tt, $J=3.9$ and 8.0Hz , OCH), 4.28-4.32(1H, m, NCH), 4.46-4.50(1H, m, NCH), 5.62(1H, bs, NHCO), 6.40(1H, bs, NHCO), 6.62(1H, t, $J=5.7\text{Hz}$, NHCO), 7.10-7.14(3H, m, Py), 7.23(1H, t, $J=6.0\text{Hz}$,

NHCO), 7.32-7.36(3H, m, Py), 7.43(1H, d, J=8.2Hz, Py), 7.56-7.61(3H, m, Py), 8.06(1H, dd, J=2.3 and 8.2Hz, Py), 8.17(1H, t, J=5.0Hz, NHCO), 8.46-8.50(3H, m, Py), 8.96(1H, d, J=2.3Hz, Py)

¹³C NMR (CDCl₃, 125MHz): δ 25.0(CH₂), 25.5(CH₂), 26.2(CH₂), 27.7(CH₂), 27.8(CH₂), 29.0(CH₂), 35.6(CH₂CO), 36.1(CH₂CO), 39.0(CH₂NH), 39.3(CH₂NH), 40.7(CH₂S), 41.0(CH₂NH), 55.6(CH₂S), 59.2(CH₂N), 60.2(CHNH), 60.7(CH₂Py), 60.8(CH₂Py), 61.0(CH₂Py), 61.8(CHNH), 67.4(CHOH), 122.1(Py), 122.8(Py), 123.2(Py), 128.5(Py), 135.6(Py), 136.5(Py), 148.0(Py), 149.0(Py), 159.3(Py), 159.4(Py), 162.6(Py), 163.9(NCON), 166.3(CONH), 173.4(CONH), 174.9(CONH)

Production Example 3-2: Solution containing the inventive zinc complex



To a phosphoric acid buffer (pH=6.86), dissolved was 200nmol of the compound obtained in Production Example 3-1 to prepare 3mM solution containing the compound, followed by addition of zinc nitrate of 2 equivalents to the solution. Thus, a solution containing the inventive zinc complex was prepared.

The solution was analyzed by the MALDI-TOF mass spectrometer (matrix: 2',4',6'-trihydroxyacetophenone), and the zinc complex was identified. A result of measurement by the MALDI-TOF mass spectrometer is shown in FIG.4. As shown in FIG.4, a molecular ion peak at 1102.3 (exact mass: 1102.27) was observed.

Experimental Example 2

The suspension (0.3mL) containing Streptavidin agarose (quantity of the bonded moiety of biotin: 60 to 120nmol/mL) and a buffer (Sigma-Aldrich Co.) was charged in a centrifugal filter unit (volume: 0.5mL, filter pore diameter: 0.22 μm). The filter unit was subjected to centrifugal separation at centrifugal acceleration of 2,000×g for 15

seconds, followed by filtration of the suspension. 5.0mM Tris-acetate buffer (pH=7.4, 0.30mL) was charged in the filter unit, then the filter unit was subjected to centrifugal separation at 2,000×g for 15 seconds and filtration for washing. This centrifugal separation and filtration step for washing was cyclically repeated for 5 times.

To the filter unit, charged was 5.0mM Tris-acetate buffer (pH=7.4, 0.30mL) dissolving 120nmol/mL of the compound obtained in Production Example 3-1 and 500nmol/mL of zinc acetate. The filter unit was brought to an equilibrium state for 5 minutes, and then, subjected to centrifugal separation at 2,000×g for 15 seconds and filtration. Next, 5.0mM Tris-acetate buffer (pH=7.4, 0.30mL) was charged in the filter unit, and subjected to centrifugal separation at 2,000×g for 15 seconds and filtration for washing. This centrifugal separation and filtration step for washing was cyclically repeated for 5 times. Further, 5.0mM Tris-acetate buffer (pH=7.4, 0.30mL) dissolving 10nmol/mL of zinc acetate was charged in the filter unit, and subjected to centrifugal separation at 2,000×g for 15 seconds and filtration for washing. This centrifugal separation and filtration step for washing was cyclically repeated for 5 times.

To the filter unit, charged was 5.0mM Tris-acetate buffer (pH=7.4, 0.30mL) dissolving non-phosphorylated peptide p60c (p60c-src peptide 521-533, 14nmol/mL) and phosphorylated peptide P-p60c (O-phosphoryl p60c-src peptide 521-533, 12nmol/mL), as samples. The filter unit was brought to an equilibrium state for 5 minutes, and then, subjected to centrifugal separation at 2,000×g for 15 seconds and filtration. The obtained filtrate was taken out as a fraction 1.

Next, 5.0mM Tris-acetate buffer (pH=7.4, 0.50M NaNO₃, 0.30mL) was charged in the filter unit, and the filter unit was subjected to centrifugal separation at 2,000×g for 15 seconds and filtration for 3 times. The filtrate obtained after the first filtration was taken out as a fraction 2, the filtrate after the second filtration was taken out as a fraction 3, and the filtrate after the third filtration was taken out as a fraction 4.

Furthermore, 1.0mM buffer containing phosphoric acid-sodium hydroxide (pH=7.4, 0.50M NaNO₃, 0.30mL) was charged in the filter unit, and the filter

unit was subjected to centrifugal separation at 2,000×g for 15 seconds and filtration for 2 times. The filtrate obtained after the first filtration was taken out as a fraction 5, and the filtrate after the second filtration was taken out as a fraction 6.

5 The contents of the peptide in the respective fractions 1 through 6 were quantitatively determined by high performance liquid chromatography (HPLC). The isolation/recovery rates of the respective peptides to the total amount are shown in Table 1.

Table 1

Fraction No.	1	2	3	4	5	6
p60c (%)	67	24	9	0	0	0
P-p60c (%)	0	0	0	0	83	17

10 Analyzing conditions according to HPLC:

column: Capcell Pak C18 type UG80, 4.6mm (diameter)×150mm
mobile phase: acetonitrile:water=14:86(v/v), 0.1%(v/v) of
trifluoroacetic acid

15 flow rate: 1 mL/min.

column temperature: 40°C

detecting method: UV 266nm

retention time: P-p60c (5.3min.), p60c (13.4min.)

20 The above results reveal that the inventive complex compound can adsorb selectively a phosphorylated peptide and use of the inventive complex compound is advantageous in selectively isolating the phosphorylated peptide in a mixed sample of the phosphorylated peptide and the non-phosphorylated peptide.

25 Comparative Example 1

30 The suspension (0.3mL) containing Streptavidin agarose (quantity of the bonded moiety of biotin: 60 to 120nmol/mL) and a buffer (Sigma-Aldrich Co.) was charged in a centrifugal filter unit (volume: 0.5mL, filter pore diameter: 0.22 μm). The filter unit was subjected to centrifugal separation at 2,000×g for 15 seconds, followed by filtration of the suspension. Then, 5.0mM Tris-acetate buffer (pH=7.4, 0.30mL) was

charged in the filter unit, and the filter unit was subjected to centrifugal separation at 2,000Xg for 15 seconds and filtration for washing. This centrifugal separation and filtration step for washing was cyclically repeated for 5 times.

5 A process similar to the process in Experiment Example 2 was conducted with use of samples equivalent to the samples used in Experimental Example 2, using a filter unit (in this Comparative Example, the inventive complex compound was not included). Thus, fractions 7 through 12 were obtained.

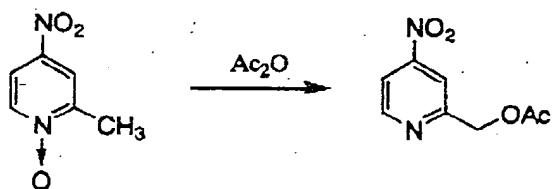
10 HPLC analysis was conducted with respect to the fractions 7 through 12 in a similar manner as Experimental Example 2. Results of the analysis are shown in Table 2.

Table 2

Fraction No.	7	8	9	10	11	12
p60c (%)	75	20	5	0	0	0
P-p60c (%)	83	17	0	0	0	0

15 The results in Table 2 reveal that separation of the phosphorylated peptide from the non-phosphorylated peptide is unsuccessful, even if a process similar to the process in Experimental Example 2 is conducted, because the inventive complex compound is not used.

Production Example 4-1: 2-Acetoxyethyl-4-nitropyridine



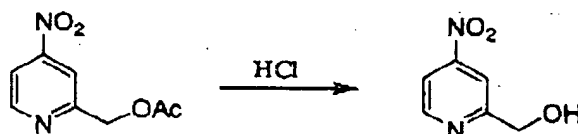
20 200mL of acetic anhydride was heated to 100°C, followed by gradual addition of 2-methyl-4-nitropyridine N-oxide (25.0g, 162mmol). After the addition, the mixture was gradually heated, and was kept at 130°C for 20 minutes. Then, the reaction mixture was cooled to 80°C, followed by dropwise addition of 200mL of ethanol to stop the reaction.

25 After the reaction mixture was concentrated, the residue was put in water of 500mL, and the pH of the mixture was adjusted to 9 by adding sodium hydrogencarbonate. After extraction with ethyl acetate twice (500 mL in

the first extraction, and 200 mL in the second extraction), the obtained organic layers were washed with 200mL of water and 200mL of brine. After concentration of the organic layer, the obtained crude product was purified by silica gel column chromatography, thereby to yield 7.68g of the target compound.

¹H-NMR (CDCl₃, 300MHz) : δ 2.23(3H, s, COCH₃), 3.36(2H, s, CH₂Py), 7.97(1H, dd, Py), 8.07(1H, d, Py), 8.90(1H, d, Py).

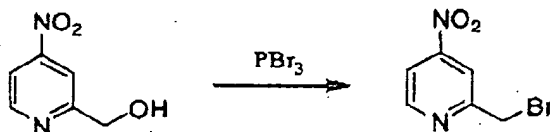
Production Example 4-2: 2-Hydroxymethyl-4-nitropyridine



To 2-acetoxymethyl-4-nitropyridine (7.68g, 39.2mmol) obtained in Production Example 4-1, added was 30mL of 10% hydrochloric acid. The mixture was reacted at 50°C for 30 minutes. After the reaction mixture was cooled, the solution was poured into 200mL of water, and the pH of the mixture was adjusted to 9 by adding sodium hydrogencarbonate. The mixture was extracted with 100mL of ethyl acetate for three times, and the obtained organic layers were concentrated. The residue obtained by the concentration was purified by silica gel column chromatography, thereby to yield 4.70g of the target compound.

¹H-NMR (CDCl₃, 300MHz) : δ 3.36(1H, t, OH), 4.94(2H, d, CH₂Py), 7.95(1H, ddt, Py), 8.09(1H, dt, Py), 8.86(1H, d, Py)

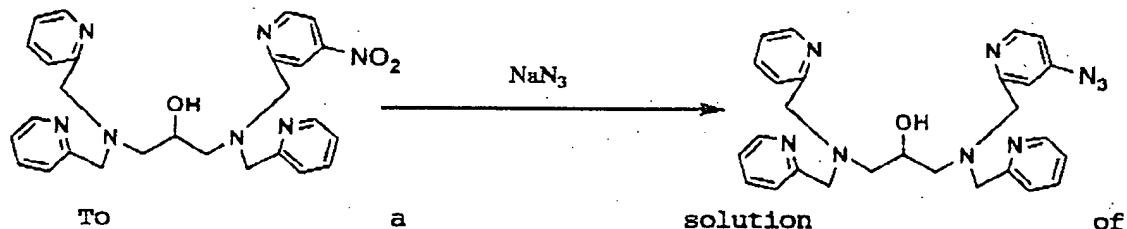
Production Example 4-3: 2-Bromomethyl-4-nitropyridine



To a suspension of 2-hydroxymethyl-4-nitropyridine (1.00g, 6.49mmol) obtained in Production Example 4-2 in dried ether (40mL), was added phosphorus tribromide (0.88g, 3.24mmol) at 0°C dropwise. After the dropwise addition, the mixture was reacted at 0°C for 2 hours, followed by reaction at 20°C for 64 hours.

CH₂Py), 3.96-4.03(1H, m, OCH), 4.07(2H, s, CH₂Py), 7.09-7.14(3H, m, Py), 7.34(3H, t, Py), 7.55-7.62(3H, m, Py), 7.80(1H, dd, Py), 8.22(1H, d, Py), 8.49-8.52(3H, m, Py), 8.76(1H, dd, Py)

5 Production Example 4-5: N,N,N'-Tri(2-pyridylmethyl)-N'-(4-azide-2-pyridylmethyl)-1,3-diaminopropane-2-ol

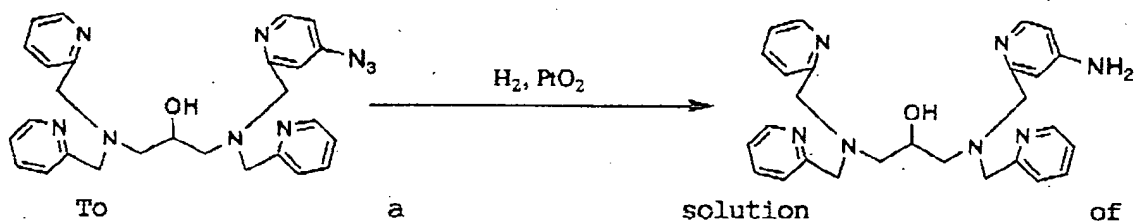


N,N,N'-tri(2-pyridylmethyl)-N'-(4-nitro-2-pyridylmethyl)-1,3-diaminopropane-2-ol (1.01g, 2.00mmol) obtained in Production Example 4-4 in dried tetrahydrofuran (17mL), was added sodium azide (156mg, 2.40mmol) in water (3.3mL) dropwise at room temperature. The reaction mixture was heated to 50°C, and reacted at the same temperature, i.e., 50°C, for 17 hours.

After the mixture was cooled, the aqueous layer was extracted with 10mL of chloroform twice. The extracts were collected and concentrated, and the resulting crude product obtained was purified by silica gel column chromatography, thereby to yield 980mg of the target compound.

¹H-NMR (CDCl₃, 300MHz) : δ 2.58-2.74(4H, m, NCH₂), 3.83-3.92(8H, m, CH₂Py), 3.92-4.01(1H, m, OCH), 6.74(1H, dd, Py), 7.09-7.14(3H, m, Py), 7.20(1H, d, Py), 7.32-7.36(3H, m, Py), 7.55-7.60(3H, m, Py), 8.39(1H, d, Py), 8.49-8.52(3H, m, Py)

Production Example 4-6: N,N,N'-Tri(2-pyridylmethyl)-N'-(4-amino-2-pyridylmethyl)-1,3-diaminopropane-2-ol

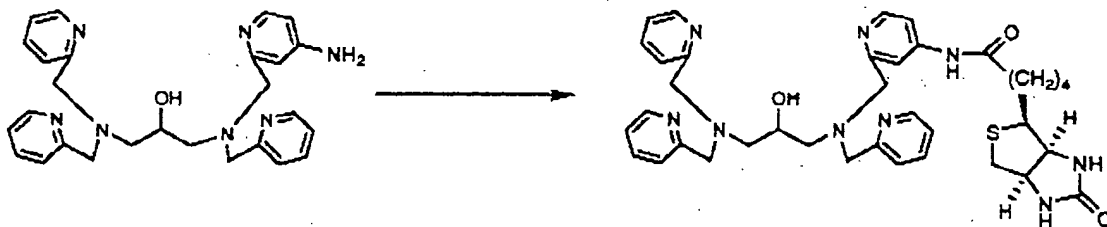


N,N,N'-tri(2-pyridylmethyl)-N'-(4-azide-2-pyridylmethyl)-1,3-diaminopropane-2-ol (980mg, 1.98mmol) obtained in Production Example 4-5 in ethanol

(20mL), was added 100mg of platinum oxide. The mixture was reacted under hydrogen pressure of 30kPa for 2 hours. After the catalyst (platinum oxide) was removed by filtration, the filtrate was concentrated, and the resulting crude product was purified by silica gel column chromatography, thereby to yield 690mg of the target compound.

¹H-NMR (CDCl₃, 300MHz) : δ 2.43-2.71(4H, m, NCH₂), 3.66-3.92(8H, m, CH₂Py), 3.92-4.01(1H, m, OCH), 6.35(1H, dd, Py), 6.63(1H, d, Py), 7.08-7.14(3H, m, Py), 7.27-7.39(3H, m, Py), 7.54-7.62(3H, m, Py), 8.10(1H, d, Py), 8.49-8.50(3H, m, Py)

Production Example 4-7: N,N,N'-Tri(2-pyridylmethyl)-N'-(4-D-biotinamido-2-pyridylmethyl)-1,3-diaminopropane-2-ol



62.4mg of D-biotin (0.26mmol) was suspended into 5mL of dichloromethane, followed by addition of 5.2mg of 4-dimethylaminopyridine (0.04mmol) and 35.8μL of triethylamine (0.26mmol) at room temperature. To this mixture, was added a solution of N,N,N'-tri(2-pyridylmethyl)-N'-(4-amino-2-pyridylmethyl)-1,3-diaminopropane-2-ol (100mg, 0.21mmol) obtained in Production Example 4-6 in dichloromethane (2mL) dropwise at room temperature. After the dropwise addition, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (49.0mg, 0.26mmol) was added at room temperature. The reaction mixture was heated, and reacted under reflux for 5 hours. To the reaction mixture, were added D-biotin (6.0mg, 0.02mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (10.0mg, 0.05mmol), and 2mL of dichloromethane, followed by reaction under reflux for 6 hours.

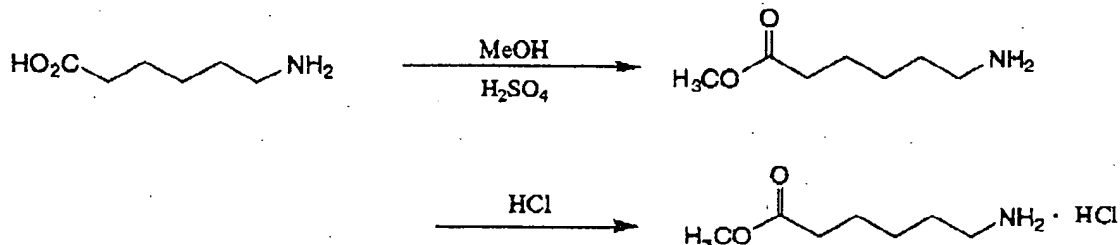
After the reaction mixture cooled, the mixture was poured into 40mL of water. After extraction with 30mL of chloroform for three times, the organic layers were washed with 30mL of water twice and 30mL of brine. After the organic layer was dried over anhydrous magnesium sulfate and

concentrated, the resulting crude product was purified by HPLC, thereby to yield 62.5mg of the target compound.

¹H-NMR (CDCl₃, 300MHz) : δ 1.37-1.45(2H, m, CH₂), 1.53-1.71(4H, m, CH₂), 2.19-2.25(2H, m, COCH₂), 2.62-2.75(5H, m, NCH₂, SCH₂), 2.87-2.93(1H, dd, SCH₂), 3.09-3.15(1H, m, SCH), 3.67-3.80(9H, m, OCH, NCH₂Py), 4.28-4.32(1H, m, NCH), 4.48-4.52(1H, m, NCH), 5.15-5.40(2H, m, NHCO) 5.89(1H, bs, NHCO) 6.48-6.50(1H, m, Py), 6.72-6.76(1H, m, Py), 7.13-7.17(3H, m, Py), 7.27-7.29(1H, m, Py), 7.37(2H, d, Py), 7.56-7.66(3H, m, Py), 8.00(1H, dd, Py), 8.49-8.50 (3H, m, Py)

This compound was examined by the MALDI-TOF mass spectrometer using α -cyano-4-hydroxycinnamic acid (CHCA) as a matrix. A result of measurement is shown in FIG.5. As is obvious from FIG.5, a molecular ion peak 696.2 of proton adduct ion (M⁺+1) was observed.

15 Production Example 5-1: Methyl 6-aminohexanoate hydrochloride



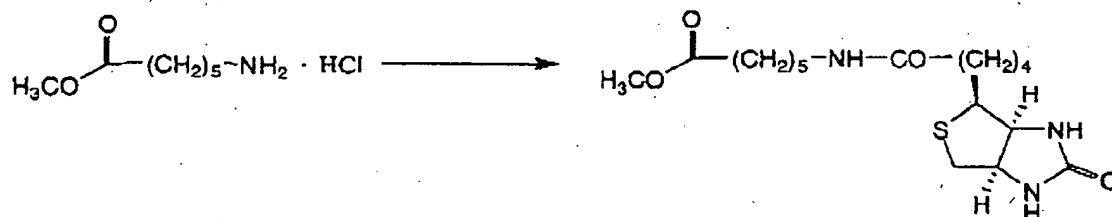
To a suspension of 6-aminohexanoic acid (10.0g, 76.2mmol) in methanol (300mL), was added 5.51g of concentrated sulfuric acid. The mixture was heated and reacted for 8 hours, while the water formed along with the progress of esterification distilled off with methanol and 60mL of methanol was added four times on cue. The reaction mixture was cooled, and the pH of the mixture was adjusted to 6.2 by adding 28% methanol solution of sodium methoxide. The solvent was distilled off, the residue was added to 500mL of water, and the pH of the mixture was adjusted to 10.3 by adding sodium carbonate. After extraction with 350mL of dichloromethane for three times, the extract was dried over anhydrous magnesium sulfate. Thereafter, the solvent was distilled off, thereby to yield methyl 6-aminohexanoate.

The methyl 6-aminohexanoate was dissolved in 40mL of dioxane, followed by addition of 7.1mL of 4N-hydrochloride solution of dioxane. After the mixture was stirred at room temperature for 30 minutes, the solvent was

distilled off, followed by addition of ether to the residue. The precipitated crystals were isolated by filtration and dried under reduced pressure to yield 3.3g of the target compound.

¹H-NMR (DMSO-d₆, 300MHz) : δ 1.26-1.36(2H, m, CH₂), 1.48-1.61(4H, m, CH₂), 2.31(2H, t, CH₂CO), 2.73(2H, t, CH₂N), 3.59(3H, s, CH₃), 8.1(3H, bs, NH₃)

Production Example 5-2: 5-Carbomethoxypentyl-D-biotinamide

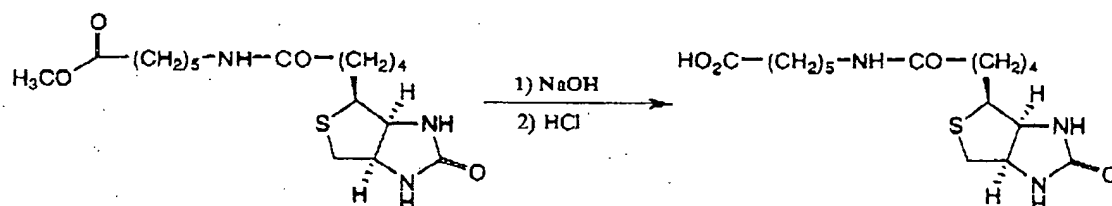


To a solution of methyl 6-aminohexanoate hydrochloride (1.00g, 5.50mmol) obtained in Production Example 5-1 in dimethylformamide (40mL), were added triethylamine (1.20g, 11.8mmol) and 4-dimethylaminopyridine (137mg, 1.1mmol). Furthermore, were added D-biotin (1.34g, 5.50mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (1.42g, 7.42mmol). The mixture was reacted at temperature from 35 to 45°C for 23 hours.

The reaction mixture was poured into 300mL of water, and extracted with 150mL of chloroform for 5 times. After the extracts were dried over anhydrous magnesium sulfate and the solvent was distilled off. The resulting crude product was purified by silica gel column chromatography, thereby to yield 1.04g of the target compound.

¹H-NMR (DMSO-d₆, 300MHz) : δ 1.18-1.65(12H, m, CH₂), 2.04(2H, t, CH₂CO), 2.28(2H, t, CH₂CO), 2.57(1H, d, CH₂S), 2.82(1H, dd, CH₂S), 3.00(1H, dt, NHCO), 3.06-3.12(1H, m, CH₂S), 3.58(3H, s, OCH₃), 4.09-4.15(1H, m, CHN), 4.28-4.32(1H, m, CHN), 6.35(1H, bs, NHCO), 6.41(1H, bs, NHCO), 7.72(1H, t, NHCO)

Production Example 5-3: 5-Carboxypentyl-D-biotinamide

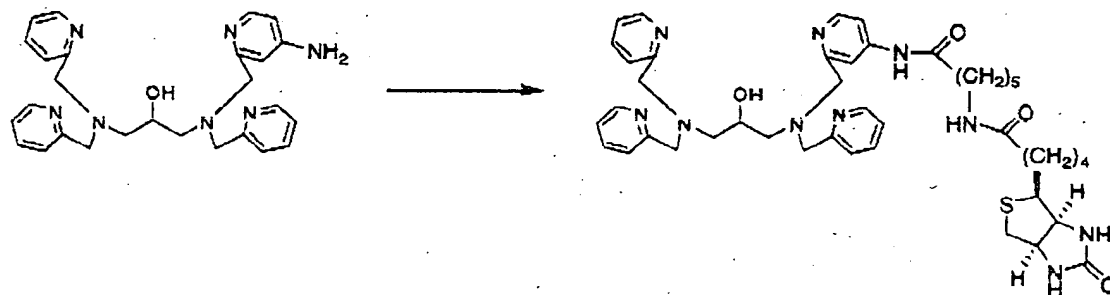


To a mixture of 25mL of tetrahydrofuran and 25mL of methanol, was dissolved 5-carbomethoxypentyl-D-biotinamide (800mg, 2.15mmol) obtained in Production Example 5-2, followed by addition of a solution of sodium hydroxide (2.33g, 58.3mmol) in water (12.5mL) dropwise at room temperature. After the addition was completed, the mixture was heated to 40°C and reacted for 2 hours.

After cooling, the reaction mixture was concentrated, followed by addition of 60mL of water. Then, 1N hydrochloric acid was added, and the pH of the mixture was adjusted to 2.0. The precipitated crystals were isolated by filtration and washed with water. The obtained crystals were dried in vacuo, thereby to yield 724mg of the target compound.

¹H-NMR (DMSO-d₆, 300MHz) : δ 1.17-1.67(12H, m, CH₂), 2.04(2H, t, CH₂CO), 2.19(2H, t, CH₂CO), 2.57(1H, d, CH₂S), 2.82(1H, dd, CH₂S), 3.00(1H, dt, NHCO), 3.06-3.12(1H, m, CH₂S), 4.13(1H, t, CHN), 4.30(1H, t, CHN), 6.36(1H, bs, NHCO), 6.43(1H, bs, NHCO), 7.74(1H, t, NHCO), 11.95(1H, bs, CO₂H)

Production Example 5-4: N,N,N'-Tri(2-pyridylmethyl)-N'-[4-(6-D-biotinamidohexacarboxyamido)-2-pyridylmethyl]-1,3-diaminopropane-2-ol



To a solution of 5-carboxypentyl-D-biotinamide (99.0mg, 0.27mmol) obtained in Production Example 5-3 in dried dimethylformamide, were added triethylamine (38.1μL, 0.27mmol) and 4-dimethylaminopyridine (5.7mg, 0.04mmol), followed by addition of a solution of N,N,N'-tri(2-pyridylmethyl)-N'--(4-amino-2-pyridylmethyl)-1,3-diaminop

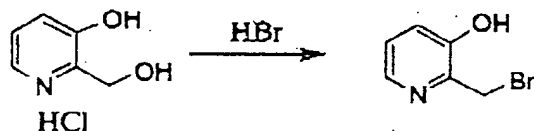
ropane-2-ol (100mg, 0.21mmol) obtained in Production Example 4-6 in dried
 dimethylformamide (1mL) dropwise. Further, was added
 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (51.7mg,
 0.27mmol). The mixture was reacted at 40°C for 24 hours. To the reaction
 5 mixture, added were 8.9 μ L of triethylamine (0.06mmol), 22.8mg of
 5-carboxypentyl-D-biotinamide (0.06mmol) and 12.2mg of
 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.06mmol).
 The mixture was reacted at 40°C for 6 hours.

10 After the reaction mixture was cooled, the mixture was poured into 150mL
 of water, followed by addition of sodium chloride to attain saturation.
 After extraction with 40mL of chloroform for 5 times, the organic layers
 were washed with 150mL of water twice and 150mL of brine. After extract
 was dried over anhydrous magnesium sulfate, the solvent was distilled off.
 The crude product obtained was purified by HPLC, thereby to yield 37.5mg
 15 of the target compound.

¹H-NMR (CDCl₃, 300MHz) : δ 1.30-1.80(12H, m, CH₂), 2.14-2.25(4H, m,
 COCH₂), 2.60-2.76(5H, m, NCH₂, CH₂S), 2.87(1H, dd, CH₂S), 3.10-3.25(3H,
 m, CH₂NHCO, SCH), 3.68-3.82(9H, m, NCH₂Py, OCH), 4.28-4.33(1H, m, NCH),
 4.45-4.52(1H, m, NCH), 5.27(1H, bs, NHCO), 5.50(1H, bs, NHCO),
 20 5.60(1H, bs, NHCO) 6.22(1H, bs, NHCO), 6.52-6.57(1H, m, Py),
 6.72-6.75(1H, m, Py), 7.16(3H, t, Py), 7.27-7.30(1H, m, Py), 7.37(2H,
 d, Py), 7.57-7.66(3H, m, Py), 7.99(1H, d, Py), 8.46-8.52(3H, m, Py)

This compound was measured by the MALDI-TOF mass spectrometer using
 CHCA as a matrix. A result of measurement is shown in FIG.6. As is obvious
 25 from FIG.6, a molecular ion peak 809.5 of proton adduct ion (M⁺+1) was
 observed

Production Example 6-1: 2-Bromomethyl-3-hydroxypyridine



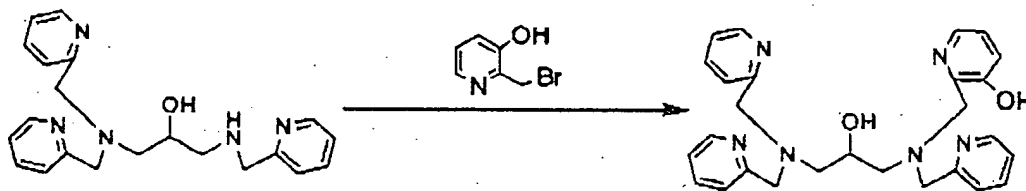
30 To 25% acetic acid solution of hydrogen bromide (12.8g, 39.6mmol), was
 added 3-hydroxy-2-hydroxymethylpyridine hydrochloride (5.0g, 54.7mmol).
 The mixture was reacted at 110°C for 1 hour. Furthermore, was added 25%

acetic acid solution of hydrogen bromide (4.90g, 15.1mmol) dropwise, and the mixture was reacted for 2 hours.

After cooling, the reaction mixture was poured into 50mL of water, and the pH of the mixture was adjusted to 8 by adding saturated aqueous sodium hydrogencarbonate solution. After extraction with 50mL of dichloromethane for four times, the dichloromethane layers were concentrated. The crude product after the concentration was purified by silica gel column chromatography, thereby to yield 1.36g of the target compound.

¹H-NMR (CDCl₃, 300MHz) : δ 5.31(2H, s, CH₂Br), 7.20-7.32(2H, m, Py), 8.18-8.21(1H, m, Py), 8.55(1H, bs, OH).

Production Example 6-2: N,N,N'-Tri(2-pyridylmethyl)-N'-(3-hydroxy-2-pyridylmethyl)-1,3-diaminopropane-2-ol

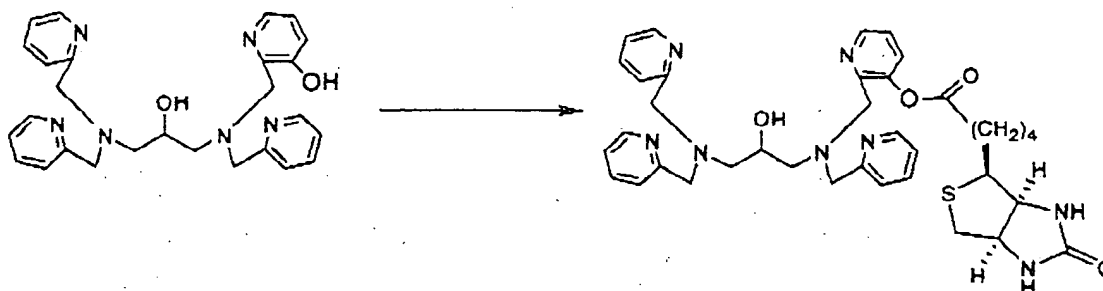


In dimethylformamide (15mL), were dissolved N,N,N'-tri(2-pyridylmethyl)-1,3-diaminopropane-2-ol (1.00g, 2.75mmol) obtained in Production Example 1-2 and 2-bromomethyl-3-hydroxypyridine (633mg, 3.37mmol) obtained in Production Example 6-1, followed by addition of potassium carbonate (760mg, 5.50mmol). The mixture was reacted at 50°C for 2 hours and cooled.

The reaction mixture was poured into 100mL of water, and the pH of the mixture was adjusted to 8 by adding 1N hydrochloric acid. After extraction with 100mL of ethyl acetate for three times, the organic layers were washed with 100mL of water and 100mL of brine, and dried over anhydrous sodium sulfate. The obtained crude product was purified and collected by HPLC, thereby to yield 660mg of the target compound.

¹H-NMR (CDCl₃, 300MHz) : δ 2.51-2.69(4H, m, NCH₂), 3.71-4.08(9H, m, CH₂Py, OCH), 7.05-7.14(4H, m, Py), 7.15-7.19(1H, t, Py), 7.23-7.28(3H, m, Py), 7.55(2H, dt, Py), 7.64(1H, dt, Py), 7.91(1H, dd, Py), 8.48-8.55(3H, m, Py)

Production Example 6-3: N,N,N'-Tri(2-pyridylmethyl)-N'-(3-D-biotinyloxy-2-pyridylmethyl)-1,3-diaminopropane-2-ol

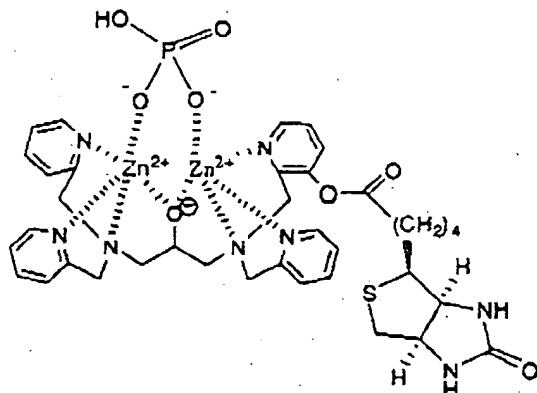


To a solution of N,N,N'-tri(2-pyridylmethyl)-N'-(3-hydroxy-2-pyridylmethyl)-1,3-diaminopropane-2-ol (100mg, 0.21mmol) obtained in Production Example 6-2 in dried dimethylformamide (5mL), was added sodium hydride (dispersion in mineral oil) (9.0mg, 0.23mmol) at 3°C. After the temperature was returned to room temperature, the mixture was reacted at room temperature for 30 minutes, followed by addition of a solution of N-succinimidyl D-biotinate (79.8mg, 0.23mmol) in dried dimethylsulfoxide (1mL) dropwise.

After the dropwise addition, the mixture was reacted at room temperature for 3.5 hours and poured into 100mL of water. After extraction with 300mL of chloroform for three times, the chloroform layers were dried over anhydrous sodium sulfate. After the solvent was distilled off, the crude product was collected and purified by HPLC, thereby to yield 63.6mg of the target compound.

¹H-NMR (CDCl₃, 300MHz) : δ 1.43-1.56(2H, m, CH₂), 1.60-1.85(4H, m, CH₂), 2.53(2H, t, COCH₂), 2.58-2.68(4H, m, NCH₂), 2.71-2.77(1H, m, SCH₂), 2.91(1H, dd, SCH₂), 3.13-3.19(1H, m, SCH), 3.76-3.99(9H, m, OCH, NCH₂Py), 4.28-4.36(1H, m, NCH), 4.47-4.53(1H, m, NCH), 5.16(1H, bs, NHCO) 5.93(1H, d, NHCO) 7.08-7.14(3H, m, Py), 7.20(1H, dd, Py), 7.33-7.42(4H, m, Py), 7.52-7.62(3H, m, Py), 8.42(1H, dd, Py), 8.51(3H, m, Py)

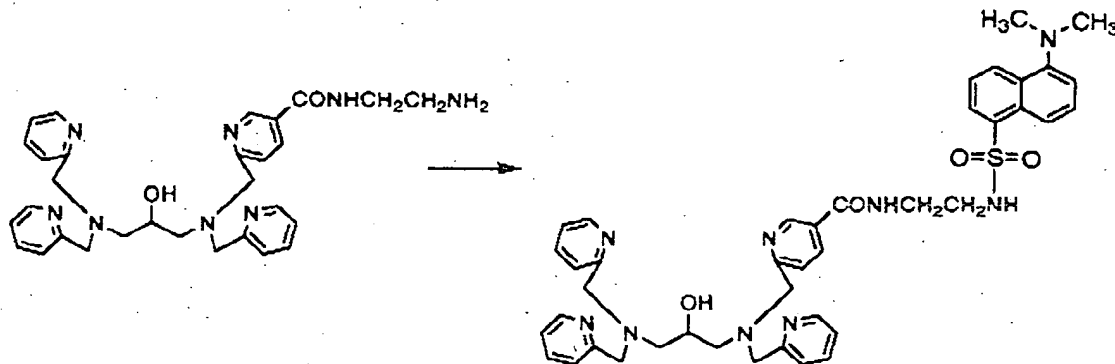
Production Example 6-4: Solution containing the inventive zinc complex



C₃₇H₄₄N₈O₈PSZn₂
Mol Wt: 922.61
Exact Mass: 919.13

To an acetonitrile solution (66.7 μ L) containing 200nmol of N,N,N'-tri(2-pyridylmethyl)-N'-(3-D-biotinyloxy-2-pyridylmethyl)-1,3-diaminopropane-2-ol obtained in Production Example 6-3, added was 40.1 μ L of 100mM zinc nitrate aqueous solution, followed by addition of 560 μ L of phosphoric acid buffer (pH=6.86). The solution was analyzed by the MALDI-TOF mass spectrometer by using 2',4'-6'-trihydroxyacetophenone (THAP) as a matrix, and the zinc complex was identified. A result of measurement by the MALD-TOF mass spectrometer is shown in FIG.7. As shown in FIG.7, a molecular ion peak at 919.1 (exact mass: 919.13) was observed.

Production Example 7-1: N,N,N'-Tri(2-pyridylmethyl)-N'-[5-N''-(2-dansylaminoethyl)carbamoyl-2-pyridylmethyl]-1,3-diaminopropane-2-ol

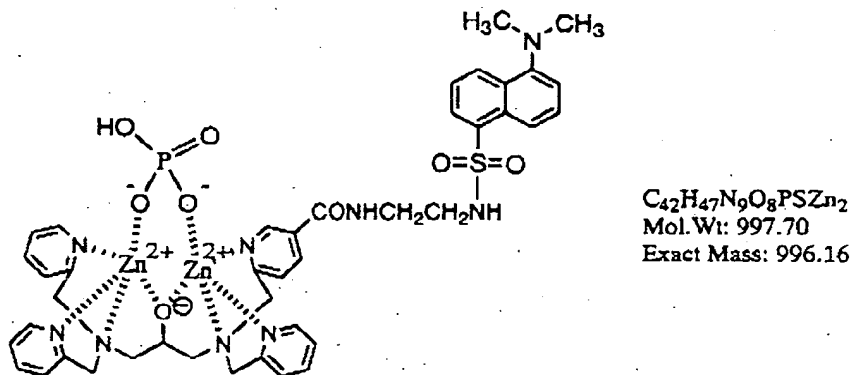


In dried acetonitrile (9mL), was dissolved N,N,N'-tri(2-pyridylmethyl)-N'-[5-N''-2-(aminoethyl)carbamoyl-2-pyridylmethyl]-1,3-diaminopropane-2-ol (1.1g, 2.1mmol) obtained in Production Example 1-4, followed by addition of dansyl chloride (840mg, 3.1mmol). The reaction mixture was heated and reacted at 50°C for 3 hours. After

the reaction mixture was concentrated, the crude product was purified by silica gel column chromatography, thereby to yield 1.30g of the target compound.

- ¹H-NMR (CDCl₃, 500MHz) : δ 2.57(1H, dd, CH₂), 2.60(1H, dd, CH₂),
 5 2.67(1H, dd, CH₂), 2.69(1H, dd, CH₂), 2.85(6H, s, NCH₃), 3.17(2H, dd, NCH₂CH₂N), 3.50(2H, dd, NCH₂CH₂N), 3.88(8H, dd, NCH₂Py), 3.83-3.92(1H, m, OCH), 6.31(1H, m, SO₂NH), 7.11(1H, d, Ar), 7.13(3H, ddd, Py), 7.35(3H, d, Py), 7.36(1H, d, Py), 7.42(1H, m, CONH), 7.46(1H, dd, Ar), 7.49(1H, dd, Ar), 7.60(2H, dt, Py), 7.60(1H, dt, Py), 7.90(1H, dd, Py), 8.23(1H, dd, Ar), 8.27(1H, d, Ar), 8.49(2H, ddd, Py), 8.50(1H, ddd, Py), 8.51(1H, dd, Ar), 8.80(1H, d, Py)
 10 ¹³C-NMR (CDCl₃, 125MHz) : 39.9(NCH₂CH₂N), 42.7(NCH₂CH₂N), 45.3(NCH₃), 59.0(CH₂), 59.1(CH₂), 60.6(CH₂Py), 60.7(CH₂Py), 61.3(CH₂Py), 67.1(CHO), 115.2(Ar), 118.6(Ar), 122.1(Py), 122.8(Py), 123.2(Ar), 123.2(Py), 128.1(Ar), 128.5(Ar), 129.5(Ar), 129.6(Ar), 129.9(Py), 130.6(Ar), 134.5(Ar), 135.4(Py), 136.5(Py), 136.6(Py), 147.6(Py), 148.9(Py), 149.0(Py), 152.0(Ar), 159.1(Py), 159.1(Py), 162.8(Py), 166.2(CONH)

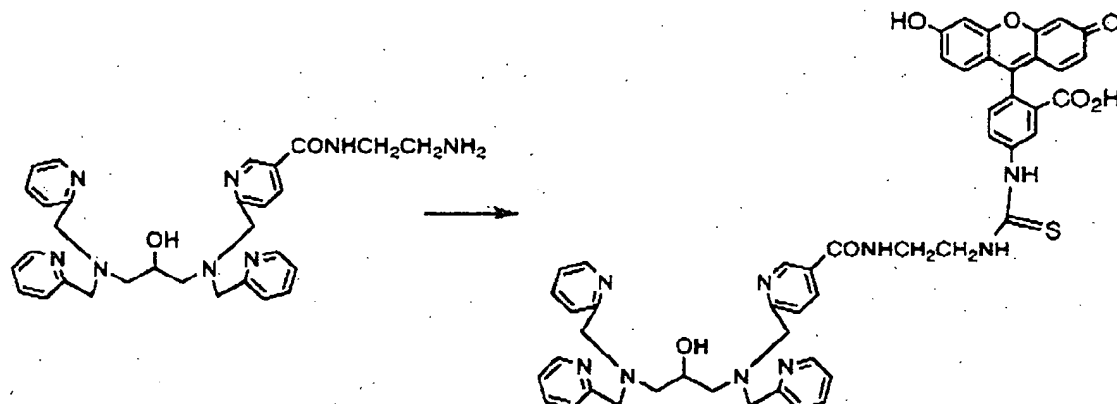
- 20 Production Example 7-2: Solution containing the inventive zinc complex



- In a phosphoric acid buffer (pH=6.86), dissolved was 200nmol of N,N,N'-tri(2-pyridylmethyl)-N'-[5-N'-(2-dansylaminoethyl)carbamoyl-2-pyridylmethyl]-1,3-diaminopropane-2-ol obtained in Production Example 7-1 to prepare 3mM solution, followed by addition of zinc nitrate of 2
 25 equivalents to the solution. The solution was analyzed by the MALDI-TOF mass spectrometer by using THAP as a matrix, and the zinc complex was identified. A result of measurement by the MALDI-TOF mass spectrometer

is shown in FIG.8. As shown in FIG.8, a molecular ion peak at 996.2 (exact mass: 996.16) was observed.

Production Example 8-1: N,N,N'-Tri(2-pyridylmethyl)-N'-[5-N''-(2-N-5-fluoresceinythioureidoethyl)carbamoyl-2-pyridylmethyl]-1,3-diaminopropane-2-ol



In dried dimethylformamide (150mL), was dissolved N,N,N'-tri(2-pyridylmethyl)-N'-[5-N''-(2-aminoethyl)carbamoyl-2-pyridylmethyl]-1,3-diaminopropane-2-ol (950mg, 1.76mmol) obtained in Production Example 1-4, followed by addition of dried pyridine (38mL). Then, a solution of fluorescein-5-isothiocyanate (750mg, 1.92mmol) in dried dimethylformamide (150mL) was added dropwise, and the mixture was reacted at room temperature for 3 hours. After the reaction mixture was concentrated, the crude product was purified by silica gel column chromatography, thereby to yield 961mg of the target compound.

¹H-NMR (DMSO-d₆, 300MHz) : δ 2.33-2.43(2H, m, CH₂), 2.50-2.61(2H, m, CH₂), 3.38-3.57(4H, m, NCH₂CH₂N), 3.67-3.92(9H, m, NCH₂Py, CHO), 6.52-6.67(6H, m, Ar), 7.16-7.23(3H, m, Py), 7.17(1H, d, Ar), 7.34-7.41(3H, m, Py), 7.52(1H, d, Py), 7.69(3H, dt, Py), 7.68-7.77(1H, m, Ar), 8.14(1H, dd, Py), 8.24(1H, d, Ar), 8.41-8.46(3H, m, Py), 8.78(1H, m, NHCO), 8.92(1H, d, Py)

Experimental Example 3 10% Native-polyacrylamide gel electrophoresis

First, prepared were gels for electrophoresis, pH buffer for electrophoresis, and a coloring solution for dissolving samples under the same condition as in Experimental Example 1.

Next, 2 μ g each of 1: Bovine Serum Albumin, 2: Human Serum Albumin, 3: carbonic anhydrase, 4: β -galactosidase, 5: α -casein (phosphorylated), 6: α -casein (de-phosphorylated), 7: β -casein (phosphorylated), 8: β -casein (de-phosphorylated), 9: pepsin (phosphorylated), and 10: pepsin (de-phosphorylated) was dissolved in the coloring solution to prepare samples. The respective samples were plotted on the gel. Then, a constant electric current of 40mA was applied until the coloring marker was flowed.

The obtained gel was immersed in a 10mM Tris-acetate buffer (pH=7.4) containing 1 μ M of N,N,N'-tri(2-pyridylmethyl)-N'-[5-N'-(2-N-5-fluoresceinylthioureidoethyl)carbamoyl-2-pyridylmethyl]-1,3-diaminopropane-2-ol obtained in Production Example 8-1 and 100 μ M zinc acetate for 30 minutes. Thereafter, the gel was taken out from the solution. This gel was washed with 100mL of 10mM Tris-acetate buffer (pH=7.4) for 10 minutes twice. Thereafter, a fluorescent image of the gel was photographed by using a fluoro image analyzer FLA-5000 (Fuji Photo Film Co., Ltd.) at an excitation wavelength of 473nm with use of a fluorescence detecting filter of 510nm. Then, the gel was dyed with Coomassie brilliant blue according to a conventional dying process, and the dyed gel was photographed. The gel dyed with the zinc-complex-containing solution is referred to as "gel A", and the gel dyed with Coomassie brilliant blue is referred to as "gel B", both of which are shown in FIG.9.

As is obvious from FIG.9, in case that being dyed according to the conventional dyeing process, all the peptides were dyed as shown by the gel B. On the other hand, only 5: α -casein (phosphorylated), 7: β -casein (phosphorylated) and 9: pepsin (phosphorylated) to all of which the phosphoric acid has been bonded were identified exclusively, as shown by the gel A which was treated with the solution containing the inventive zinc complex. Thus, it is clear that the inventive method is advantageous in determining phosphorylated peptides exclusively in samples derived from living organisms.

This application is based on Japanese Patent Application No.2003-56068 filed on March 3, 2003, No.2003-113707 filed on April 18, 2003 and No.2003-356934 filed on October 16, 2003, the contents

of which are hereby incorporated by references.

Although the present invention has been fully described by way of example, it is to be understood that various changes and modifications will be apparent to those skilled in the art.

- 5 Therefore, unless otherwise such changes and modifications depart from the scope of the present invention hereinafter defined, they should be construed as being included therein.